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VITAMIN B_{12}

Microbiological Assay Methods and Distribution in Selected Foods

Home Economics Research Report No. 13

Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

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By Harold Lichtenstein, Aram Beloian, and Elizabeth W. Murphy

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Human Nutrition Research Division
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UNITED STATES DEPARTMENT OF AGRICULTURE
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VITAMIN B_{12}—MICROBIOLOGICAL ASSAY METHODS AND DISTRIBUTION IN SELECTED FOODS

By Harold Lichtenstein, Aram Beloian, and Elizabeth W. Murphy, *Human Nutrition Research Division, Agricultural Research Service*

Vitamin B_{12} is a cobalt-containing substance of high molecular weight and complex polycyclic structure (*6*, *23*)[1], with a basic configuration resembling that of hemoglobin and chlorophyll (fig. 1). It is a known dietary requirement for the growth of a variety of animals (*7*, *27*, *32*, *38*, *40*). In humans its requirement in the diet is evidenced by the appearance of subclinical deficiency symptoms in adults who have subsisted on strict vegetarian diets for extended periods (*52*). The relief of the symptoms of pernicious anemia, a disorder involving impaired intestinal absorption of vitamin B_{12}, by parenteral administration of the vitamin is also evidence of its requirement in the human diet (*51*). Its basic function remains obscure, although various physiological roles at the cellular level, such as the synthesis of deoxyribosides (*13*), the metabolism of single carbon fragments (*3*), and the incorporation of amino acids into protein (*28*), have been tentatively ascribed to vitamin B_{12}. Work on a coenzyme form of vitamin B_{12}, isolated from bacterial cells, offers promise of elucidating this basic function (*50*). Extensive discussion of the chemistry, physiology, and nutrition of vitamin B_{12} is to be found in recent reviews (*5*, *15*, *19*, *26*).

Although the published literature on vitamin B_{12} is voluminous, data therein on the vitamin B_{12} potency of foods are scattered and sparse. Furthermore, the data available were obtained by diverse methods—microbiological and biological—of wide range in precision and specificity. A need existed for a standardized method of established specificity by which the vitamin B_{12} content of a broad variety of foods could be determined.

When the work on vitamin B_{12} was begun in this laboratory, it was decided to test two methods which, of the many available, appeared most promising. One, the *Ochromonas malhamensis* method of Ford (*17*), was reported to be uniquely specific, its test organism possessing a vitamin B_{12} requirement closely parallel to that of higher animals; the other, the A.O.A.C. *Lactobacillus leichmannii* method (*4*), had been widely tested and was reported to possess ease of execution, uniformity of results, but a relative lack of specificity for vitamin B_{12}. Comparative tests of the two methods (*34*), each somewhat modified, were made in order to select the one preferable for the general purpose of the study. As the result of this comparative study, additional foods were assayed by the *L. leichmannii* method alone.

The proximate composition of the foods assayed for vitamin B_{12} content was determined in order to have moisture values for dry-basis calculations, and also to characterize the samples with regard to their relative contents of other nutrients.

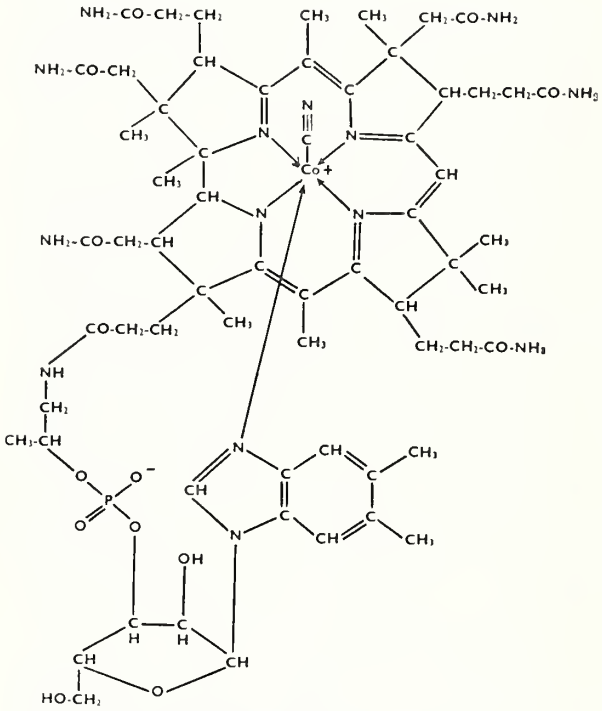

Figure 1.—VITAMIN B_{12}. (From Europäisches Symposion Hamburg 1956.)

[1] Italic numbers in parentheses refer to Literature Cited, p. 8.

This publication presents the methods used and the results of a laboratory study of the distribution of vitamin B_{12} in and the proximate composition of the edible portions of foods of animal origin common to the American diet. These foods were analyzed by standardized methods in the forms as purchased, modified by treatment as described below.

ANALYTICAL METHODS

Sampling

The majority of the samples were purchased in the Washington, D.C., market. Exceptions were lamb leg, pork loin, pork shoulder, chicken gizzard, chicken liver, and fresh egg, which were obtained from the Agricultural Research Center, Beltsville, Md., and dried whole egg, which was obtained from the processor. Newly purchased fresh foods were frozen at −18° C. until samples were prepared. Canned and dried foods other than dried egg were stored at room temperature; dried egg was stored at 5° until used.

In most instances the food samples assayed consisted of composites on an equal-weight basis of components obtained from different dealers or of different brands. Before the foods were composited, unprocessed components, such as meats, poultry, or seafood, were stripped of visible fat and waste. Most solid samples were ground three times in a meat grinder, then homogenized in a blendor, if feasible, before subsampling for analysis. Cream and cottage cheeses were stirred with bowl and spoon. Powdered samples were mixed by tumbling. The mixing of liquid or viscous samples varied with consistency. Some were poured back and forth between containers, some were blended, and others were agitated with a household-type mixer. Ground and mixed subsamples were sealed in cans and stored at −18° and −40° C. until analyzed, depending on the storage space available.

The vitamin B_{12} assays, with a few exceptions, were done on two subsample replicates of each sample, each replicate being run on a different date. Three subsample replicates were run by the *L. leichmannii* method on buttermilk, beef heart, beef liver, and ham, and by the *O. malhamensis* method on beef round steak and chicken gizzard. Proximate composition determinations were run on six subsample replicates of each sample, with three replicates run on each of two separate dates.

Methods for Proximate Composition Determination

MOISTURE

Samples were weighed in shallow, 60-mm. diameter aluminum-foil moisture dishes and dried over calcium chloride overnight (approximately 16 hours) at 50° C. in a forced-air oven. Residual moisture was removed by drying to constant weight in a vacuum oven at 50° for approximately 48 hours. Loss in weight was recorded as the moisture content.

FAT

Fat in milks and cheeses was determined by the Roese-Gottlieb method of the A.O.A.C.; the Röhrig tube was used for extraction (*4*). Fat in eggs was determined by acid hydrolysis, followed by extraction in the Röhrig tube. The fat content of all the other foods was determined on moisture-free samples by overnight continuous extraction in a Soxhlet apparatus, with petroleum ether as the solvent.

NITROGEN

The microkjeldahl method of the A.O.A.C. (*4*) was modified by using 0.02 N HCl rather than boric acid as the receiving solution and titrating with 0.02 N NaOH with methyl red as the indicator.

ASH

Samples were weighed into porcelain or vycor crucibles and dried by heating overnight at 50° C. in a forced-air oven. This method was found to be a suitable one for evaporating the water from all types of foods. For uniformity, all samples were charred at 400° overnight, as this procedure was found necessary for samples containing a high percentage of fat. Samples were then ashed for 2 to 3 hours at 600°.

Methods for the Determination of Vitamin B_{12}

Lactobacillus leichmannii METHOD

The *L. leichmannii* method used in this study was the 1955 A.O.A.C. method (*4*) with minor modifications.

Basal medium.—The components for 1 liter of double-strength basal medium are given below:

Components:	*Quantities*
L-cystine	400 mg.
DL-trytophane	400 mg.
N HCl	40 ml.
Adenine-guanine-uracil solution	20 ml.
Xanthine solution	20 ml.
Vitamin solution I	40 ml.

Components:	*Quantities*
Vitamin solution II	40 ml.
Salt solution A	20 ml.
Salt solution B	20 ml.
Asparagine solution	20 ml.
Bacto vitamin-free casamino acids	15 g.
Dextrose, anhydrous	40 g.
Sodium acetate trihydrate	32.932 g.
Ascorbic acid	4 g.
Tween 80 solution	20 ml.

Directions for the preparation of the component solutions follow.

Adenine-guanine-uracil solution.—200 mg. each of adenine sulfate, guanine hydrochloride, and uracil were dissolved in 10 ml. of warm 1+1 HCl. This solution was diluted with distilled water to 200 ml., overlaid with toluene, and stored at 5° C.

Xanthine solution.—To 200 mg. of xanthine was added 40 ml. of distilled water. The mixture was warmed to 70° C., 6 ml. of 2 + 3 NH_4OH was added, followed by agitation until solution was effected. The solution was diluted with distilled water to 200 ml., overlaid with toluene, and stored at 5°.

Vitamin solution I.—10 mg. of riboflavin, 10 mg. of thiamine hydrochloride, 0.1 mg. of biotin, 20 mg. of nicotinic acid were weighed out, dissolved in 0.02 N acetic acid, and diluted with solvent to 400 ml. This solution was overlaid with toluene and stored in a brown bottle at 5° C. For convenience, biotin was used in the form of a mixture of 100 mg. of biotin and 9.900 g. of sucrose, which had been thoroughly ground together with mortar and pestle.

Vitamin solution II.—The following materials were dissolved consecutively in 25-percent neutral ethyl alcohol: 20 mg. of p-aminobenzoic acid, 10 mg. of calcium pantothenate, 40 mg. of pyridoxine hydrochloride, 8 mg. of pyridoxamine dihydrochloride, 40 mg. of pyridoxal hydrochloride, 2 mg. of folic acid. This solution was diluted with solvent to 400 ml., and was stored without toluene in a brown bottle at 5° C.

Salt solution A.—10 mg. each of KH_2PO_4 and K_2HPO_4 were dissolved in distilled water and diluted to 200 ml. Two drops of concentrated HCl were added; the solution was overlaid with toluene and stored at 5° C.

Salt solution B.—4.0 g. of $MgSO_4 \cdot 7H_2O$, 200 mg. of NaCl, 200 mg. of $FeSO_4 \cdot 7H_2O$, 200 mg. of $MnSO_4 \cdot H_2O$ were dissolved in distilled water and diluted to 200 ml. Two drops of concentrated HCl were added; the solution was overlaid with toluene and stored at 5° C.

Asparagine solution.—2.0 g. of L-asparagine was dissolved in distilled water, diluted to 200 ml., overlaid with toluene, and stored at 5° C.

Tween 80 solution.—20 g. of tween 80 was dissolved in and diluted to 200 ml. with 95-percent ethyl alcohol. This solution was stored at 5° C.

The components of the basal medium were combined and brought into solution in the order given. Sufficient distilled water was added after the casamino acids to permit the solution of the remaining components. The pH was adjusted to 6.0 with 5 N NaOH and the volume of solution was adjusted to a liter.

If not used immediately, the basal medium was stored in screwcap polyethylene bottles by refrigeration at 5° C. for no longer than 24 hours, or at −18° for a week. Such storage resulted in no appreciable darkening of the medium and had no effect on assay organism growth response; however, storage at −18° for as long as a month resulted in progressive darkening but had a negligible effect on response.

Stock culture and inoculum culture media.—The following materials were dissolved in 700 ml. of distilled water: 7.5 g. of Bacto yeast extract, 7.5 g. of Bacto peptone, 10 g. of anhydrous dextrose, 2 g. of KH_2PO_4, and 50 ml. of tomato juice serum (clear filtrate of canned tomato juice). To this was added 5 ml. of tween 80 solution, preparation of which was described under basal medium. After the constituents of the broth were dissolved, its pH was adjusted to 6.8, it was diluted to a liter, and was then divided into two equal parts. One was dispensed in 10 ml. quantities into 16- by 150-mm. screwcap test tubes; these tubes contained the inoculum culture broth. To the remaining part was added 7.5 g. of agar; it was steamed to bring the agar into solution and dispensed into test tubes as previously described. The latter tubes contained the stock culture agar medium. All tubes were loosely capped with plastic caps, autoclaved at 120° C. (15 p.s.i.) for 15 minutes, and allowed to cool. When cooled, the caps were tightened and the tubes stored at 5°.

Maintenance of stock culture.—The stock culture of *L. leichmannii* A.T.C.C. No. 7830 was maintained by stab culture transfers into tubes of stock-culture agar medium at weekly intervals. The inoculated medium was incubated at 37° C. for 24 hours, then stored at 5° until used for transfer the following week.

Vitamin B_{12} stock solution.—Sufficient U.S.P. Cyanocobalamin Reference Standard was weighed out to contain 25 micrograms of vitamin B_{12}. This material was dissolved in 25-percent ethyl alcohol and diluted to 25 ml. It was stored at 5° C. in a glass-stoppered flask for no longer than 3 months.

Buffer-bisulfite extractant.—Shortly before use the following constituents were dissolved in distilled water and diluted to 100 ml.: 1.29 g. of Na_2HPO_4, 1.10 g. of anhydrous citric acid, and 1.00 g. of sodium metabisulfite.

Preparation of sample and vitamin B_{12} standard solution.—The sample was weighed out so that when diluted the final dilution contained approximately 0.02 millimicrogram of vitamin B_{12} per milliliter. To each gram of sample was added

25 ml. or less of buffer-bisulfite extractant, so that when fully diluted the extract contained no more than 0.006 mg. of sodium metabisulfite per milliliter. This limit was used when the extract was assayed by both the *L. leichmannii* and *O. malhamensis* methods, because *O. malhamensis* is sensitive to concentrations of bisulfite in excess of this concentration. For the *L. leichmannii* method the permissible limit of bisulfite concentration is 0.025 mg. per milliliter. The mixture of sample and extractant was autoclaved at 120° C. for 10 minutes, cooled, diluted to volume, filtered, and diluted further if necessary. The 10-minute autoclaving was satisfactory for all samples tested other than dried egg, which required 30 minutes for full and uniform extraction.

The vitamin B_{12} standard solution was prepared by the same extraction procedure as the samples. To 1 ml. of vitamin B_{12} stock solution was added 25 ml. of buffer-bisulfite extractant. The mixture was autoclaved for 10 minutes at 120° C., diluted to 200 ml., and 1 ml. of the first dilution further diluted to 250 ml., yielding a final concentration of standard solution of 0.02 millimicrogram of vitamin B_{12} per milliliter.

Suspension medium.—The suspension medium consisted of equal parts of basal medium and distilled water, dispensed in both 9 ml. and 10 ml. volumes into 16- by 150-mm. screwcap test tubes. These were prepared at the same time as assay tubes and were autoclaved with them, as described later.

Preparation of inoculum.—The day before the setting up of an assay, a transfer was made from the stock culture stab to a tube of inoculum culture broth. The inoculated broth was incubated at 37° C. for 20 hours, after which it was centrifuged in the culture tube at 2,800 r.p.m. for 10 minutes in order to sediment the cells. The supernatant liquid was decanted off and discarded, and was replaced with 10 ml. of suspension medium. The suspension was serially diluted (1 ml. of suspension added to 9 ml. of suspension medium) to a concentration of 0.0001 to 0.11 mg. dry cells per 10 ml. of suspension (*35*). The 10^{-4} dilution was almost invariably in this range. The final dilution of suspension was the inoculum.

Assay procedure.—To 16- by 150-mm. test tubes were dispensed in triplicate 1, 2, 3, 4, and 5 ml. respectively, of vitamin B_{12} standard solution. To these tubes were added distilled water to bring the volume of each up to 5 ml., followed by 5 ml. each of basal medium. This set of tubes was the assay standard, in which the vitamin B_{12} concentrations ranged from 0.02 to 0.1 millimicrogram per assay tube. To additional tubes were dispensed in triplicate 1, 2, 3, 4, and 5 ml. of each sample extract, with the distilled water and basal medium added as described for the standard. In addition to other samples, each assay included Bacto liver as a standard sample.

Six tubes were prepared to contain 5 ml. each of distilled water and basal medium; three for uninoculated blanks and three for inoculated blanks. Six additional tubes were prepared, 2 containing 10 ml. and 4 with 9 ml. each of suspension medium. The latter 6 tubes were for use in the preparation of the inoculum.

The tubes were capped with aluminum caps, autoclaved at 120° C. for 5 minutes, and cooled to room temperature rapidly by partial immersion in cold water. The assay standard, samples, and inoculated blanks were then inoculated by dispensing the inoculum dropwise into the assay tubes from a Krogh-Keys syringe fitted with a 22-gage needle and secured on a stand in a horizontal position. These, together with the uninoculated blanks, were incubated at 37° for 24 hours in a walk-in incubator. At the end of the incubation period the tubes were shaken by vibrating each tube against the free end of a 4-inch length of pressure tubing rotated by an electric motor. The contents of the tubes were transferred to cuvettes, and optical density readings of the suspensions were made on the Beckman Model B spectrophotometer at 620 millimicrons. Uninoculated blanks were read versus water, inoculated blanks versus uninoculated blanks, and all others versus inoculated blanks.

Calculation of results.—Optical density readings and corresponding vitamin B_{12} contents of the assay standard tubes were plotted on logarithmic graph paper. A straight line was drawn through the plotted points; this was the standard curve (fig. 2). By interpolation of optical density readings of sample assay tubes on the standard curve, or extrapolation short distances beyond the ends of the standard curve, estimates of their associated vitamin B_{12} concentrations were obtained. These estimates were converted to a per milliliter

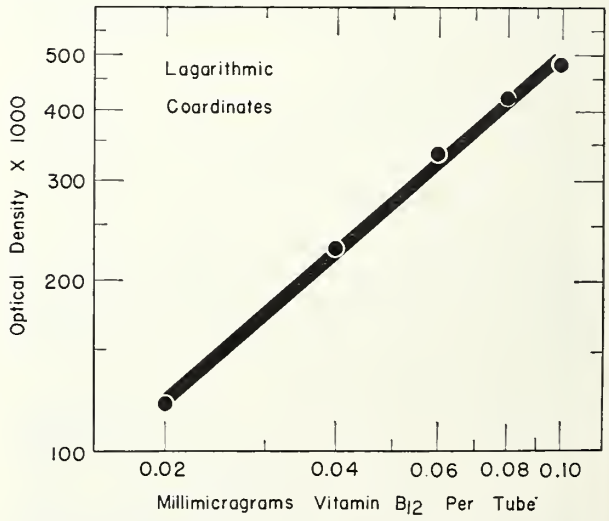

FIGURE 2.—Standard growth curve for *Lactobacillus leichmannii.*

basis and were averaged to yield a provisional mean. Individual values varying by more than ±10 percent from the provisional mean were discarded. A corrected mean calculated from the remaining values was accepted, provided that the latter consisted of three-fourths of a minimum of 12 values. From the corrected mean, sample weights, and dilutions, the vitamin B_{12} concentrations of the samples were calculated.

Ochromonas malhamensis METHOD

The *O. malhamensis* method used in this study was a modification of the method of Ford (*1*, *17*).

Basal medium.—The components for 200 ml. of fivefold-strength basal medium are given below:

Components:	*Quantities*
Bacto vitamin-free casamino acids	5 g.
Dextrose, anhydrous	10 g.
Diammonium citrate	800 mg.
KH_2PO_4	300 mg.
$MgSO_4 \cdot 7H_2O$	200 mg.
$CaCl_2$, anhydrous	150 mg.
Sodium molybdate ($NaMoO_4 \cdot 2H_2O$)	50 mg.
DL-tryptophane	100 mg.
Cystine solution	3 ml.
DL-methionine	200 mg.
Tween 80 solution	2 ml.
Metals solution	10 ml.
Vitamin solution	2 ml.
NaCN solution	0.2 ml.

Directions for the preparation of the component solutions follow.

Cystine solution.—100 mg. of L-cystine was dissolved in 3 ml. of N HCl. This was prepared just before use.

Tween 80 solution.—100 ml. of tween 80 was dissolved in 95-percent ethyl alcohol, diluted with solvent to 200 ml., and stored at 5° C.

Metals solution.—The following materials were dissolved in 900 ml. of hot distilled water in the order given: 5.75 g. of versene (sodium (di) ethylenediamine tetraacetic acid), 6 g. of $MnSO_4 \cdot H_2O$, 11 g. of $ZnSO_4 \cdot 7H_2O$, 1 g. of $FeSO_4 \cdot 7H_2O$, 300 mg. of $CoSO_4 \cdot 5H_2O$, 40 mg. of $CuSO_4 \cdot 5H_2O$, 60 mg. of H_3BO_3, and 1 mg. of KI. This solution was diluted to a liter and stored at −18° C. in a screwcap polyethylene bottle.

Vitamin solution.—1 g. of inositol, 200 mg. of choline chloride, 100 mg. of p-aminobenzoic acid, 200 mg. of thiamine hydrochloride, and 1 mg. of biotin were dissolved in 20-percent ethyl alcohol and diluted with solvent to 200 ml. This solution was stored at 5° C.

Sodium cyanide solution.—1 g. of NaCN was dissolved in distilled water and diluted to 100 ml. This solution was prepared just before use.

The components of the basal medium listed above were dissolved consecutively in 150 ml. of distilled water. After the pH was adjusted to 4.5 and the dilution to 200 ml., the basal medium was stored at −18° C. in a screwcap polyethylene bottle.

Suspension medium.—20 ml. of basal medium was mixed with 80 ml. of distilled water, dispensed in 9.5 ml. volumes into 16- by 150-mm. screwcap tubes, capped loosely, and autoclaved at 115° C. (10 p.s.i.) for 10 minutes. After the tubes were cooled, the caps were tightened, and the tubes were stored at 5° for no longer than 3 weeks.

Culture medium.—To 5 ml. of a 1 to 200 dilution of vitamin B_{12} stock solution (*see* under *L. leichmannii* method) was added 20 ml. of basal medium, and water was added to make 100 ml. This was adjusted to pH 5.5, distributed in 12 ml. portions in 50-ml. erlenmyer flasks, plugged with cotton and covered with glassine bags, and autoclaved at 115° C. (10 p.s.i.) for 15 minutes. Culture medium was stored at 5° for no longer than 3 weeks.

Maintenance of stock culture.—The stock culture of *O. malhamensis*, A.T.C.C. No. 11532, was maintained by transferring at 5-day intervals a single drop from a growing culture to a flask of culture medium previously warmed to incubator temperature. The inoculated medium was incubated at 28° to 29° C., 1 foot from a 15-watt fluorescent lamp.

Preparation of inoculum.—The inoculum culture was prepared in the same manner as the stock culture, except that it was incubated for only 4 days. Its transmittance was read in the Beckman Model B spectrophotometer at 580 millimicrons versus suspension medium; it was then adjusted by the addition of suspension medium to 70-percent transmittance. In practice, adjustment to 70-percent transmittance was effected by diluting 0.7 ml. of inoculum culture with 9.5 ml. of suspension medium. The diluted suspension, containing approximately 35 x 10^3 cells per drop, was the inoculum.

Vitamin B_{12} stock solution; buffer-bisulfite extractant; preparation of sample extract.—These were the same as described under the *L. leichmannii* method.

Preparation of vitamin B_{12} standard solution.—The procedure described under the *L. leichmannii* method was followed, with an additional step. After the stock vitamin B_{12} solution was diluted to 200 ml., this first dilution was diluted 1 ml. to 250 ml. For the *O. malhamensis* method an additional dilution was made, 5 ml. of the first dilution to 250 ml. This provided two vitamin B_{12} standard solutions, of concentrations 0.02 and 0.1 millimicrograms per milliliter.

Assay procedure.—To 25- by 50-mm. shell vials were dispensed in triplicate 1, 2, 3, and 4 ml. of 0.02 millimicrogram per milliliter of vitamin B_{12} standard solution, and 1 ml. of 0.1 millimicrogram per milliliter of vitamin B_{12} solution. To these vials was added distilled water to bring the volume of each up to 4 ml., then 1 ml. of basal medium was added to each vial. This set of vials was the

assay standard, in which the vitamin B_{12} concentrations ranged from 0.02 to 0.1 millimicrogram per vial. To additional vials were dispensed in triplicate 1, 2, 3, and 4 ml. of sample extract, distilled water, and basal medium as described for the assay standard. In addition to other samples, each assay included Bacto liver as a standard sample. Six vials were set up, each containing 1 ml. of basal medium and 4 ml. of distilled water—three for uninoculated blanks and three for inoculated blanks.

The vials were capped with aluminum caps, autoclaved at 115° C. (10 p.s.i.) for 10 minutes, allowed to cool to incubator temperature in the 28° to 29° incubator, and all except the uninoculated blanks inoculated. The inoculum was administered to vials dropwise from a Krogh-Keys syringe fitted with a 13-gage needle and clamped to a stand in a horizontal position. All vials, including the uninoculated blanks, were incubated without shaking in the dark at 28° to 29° in a walk-in incubator for 5½ days. At the end of the incubation period the cultures were steamed for 5 minutes, allowed to cool, stoppered with rubber stoppers, and then shaken by hand. The contents of the vials were transferred to cuvettes and optical density readings were made on the Beckman Model B spectrophotometer at 580 millimicrons. Uninoculated blanks were read versus water, inoculated blanks versus uninoculated blanks, and all others versus inoculated blanks.

Calculation of results.—Optical density readings and corresponding vitamin B_{12} contents of the standard assay vials were plotted on arithmetic graph paper. A curve drawn through the plotted points constituted the standard curve (fig. 3). By interpolation of optical density readings of sample assay vials on the standard curve, or extrapolation short distances beyond the ends of the curve, estimates of their associated vitamin B_{12} concentrations were obtained. These estimates were converted to a per milliliter basis and were averaged to yield a provisional mean. Individual values varying by more than ±10 percent from the provisional mean were discarded. A corrected mean calculated from the remaining values was accepted when the latter numbered 8 out of 12 values. From the corrected mean, sample weights, and dilutions, the vitamin B_{12} potencies of the samples were calculated.

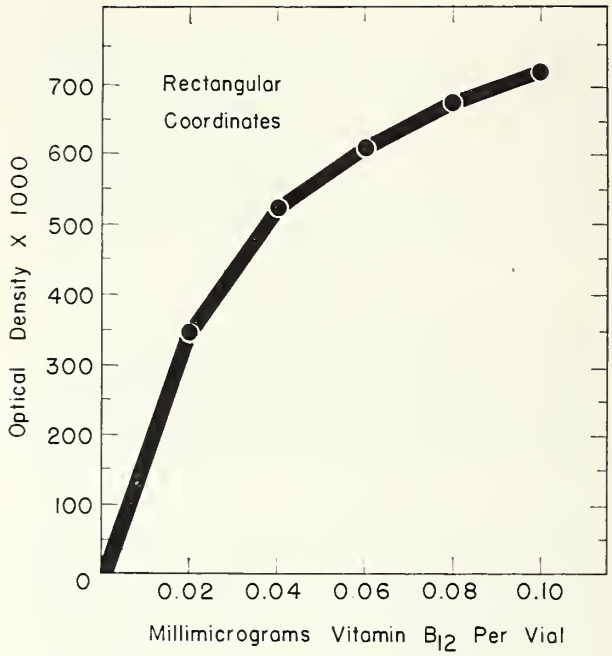

FIGURE 3.—Standard growth curve for *Ochromonas malhamensis*.

STATISTICAL EVALUATION

Precision of the Vitamin B_{12} Assays

The data from 165 subsamples of 81 food samples assayed by the *L. leichmannii* method were submitted to analysis of variance. Logarithmic transformation of the data was necessary in order to obtain homogeneity of variance. From the mean square for within samples a standard error of the mean was calculated. The latter and the appropriate t value furnished upper and lower 95-percent confidence limits applicable to each sample mean. For ease in presentation the percentage deviation of each limit from its mean was calculated. The means of the percentage deviations were +6.4 and −6.0. Similar treatment of the data from 56 subsamples of 27 food samples assayed by *O. malhamensis* method yielded mean percentage deviations of limits from sample means of +8.8 and −8.1.

The statistical analyses did not include data on imported Swiss cheese, condensed milk, frankfurters, or whole egg. These foods were analyzed for their vitamin B_{12} content subsequent to the statistical study.

Comparison of the *L. leichmannii* and *O. malhamensis* Methods

It has been well established that the *L. leichmannii* method is not specific for vitamin B_{12}. In contrast to this, the *O. malhamensis* method has been claimed to be quite specific (*8*, *9*, *31*, *39*). The claim was based largely on the results of the assay of fecal material and intestinal contents, which contain significant concentrations of vitamin B_{12} analogs. Several of these analogs elicit growth responses in the *L. leichmannii* but not in the *O. malhamensis* methods. *L. leichmannii* also responds to nucleic acid derivatives, whereas *O. malhamensis* does not (*19*).

On the basis of the reported difference in specificity, higher results might have been expected in the assay of foods by the *L. leichmannii* method. This was not borne out by a comparison of the results of the two methods (*34*), data from which are incorporated in table 1. Parallel assays by both methods on the same samples, in many instances on the identical extracts, yielded results that appeared generally to be of the same order of magnitude. Analyses of variance of the logarithms of the potency data indicated that the overall means of the two methods were significantly different at the 5-percent level. The standard error of the difference between the means of the two methods was calculated from the pooled sums of squares for within samples. This standard error was used for *t* tests on individual samples. Where *t* tests indicated significantly divergent values, mean *O. malhamensis* values were higher than the *L. leichmannii* values. In 15 of the 27 samples tested the *O. malhamensis* values were significantly higher than the *L. leichmannii* values, with mean differences of 13 percent or more. Yoghurt, alone, gave a significantly higher value by the *L. leichmannii* than by the *O. malhamensis* method, the difference being 26 percent.

In foods containing bacterial growth products and in organ meats, where the nonspecificity of the *L. leichmannii* method was expected to be evident if nowhere else, insufficient nonspecific activity was present to interfere with the *L. leichmannii* method, except with a single food, yoghurt. A reasonable explanation for the significantly higher *O. malhamensis* values is that substances other than vitamin B_{12} which are stimulatory to *O. malhamensis* occur in the foods analyzed.

A Bacto liver standard sample was assayed with each set of food samples analyzed. In 61 replicates by the *L. leichmannii* method and in 24 by the *O. malhamensis* method, the latter method also yielded a higher mean vitamin B_{12} value (table 1).

DISTRIBUTION OF VITAMIN B_{12} IN FOODS

Of all the foods tested (table 1), those highest in vitamin B_{12} occurred among the organ meats. Beef and lamb livers had moist-basis values of 116 and 104 and beef and lamb kidneys, values of 38 and 63 micrograms per 100 grams, respectively. Beef brain and heart values were somewhat lower than these, but considerably higher than those of ordinary muscle meats. Chicken liver and heart values were higher than those of poultry muscle meats, but lower than those of organs of the mammals tested. Of the organ meats, beef thymus was scarcely higher in vitamin B_{12} than some of the muscle cuts. Other foods found to be rich in vitamin B_{12} were egg yolk and certain of the shellfish. Clam, oyster, and crab had values comparable to organ meats. Moderately high in vitamin B_{12} were fresh salmon and rockfish and canned salmon and sardines; the vitamin B_{12} values for these sardines were almost as high as those for some organ meats.

Of the dairy products assayed, evaporated milk and yoghurt had extremely low and condensed milk moderately low vitamin B_{12} potencies. This would have been unexpected considering that the processes by which these products are prepared from whole milk involve concentration. However, a study on the processing of raw whole milk into condensed milk and evaporated milk (*10*) showed that vitamin B_{12} losses incurred during processing amounted to 40 and 90 percent, respectively, whereas less than 10-percent loss resulted from pasteurization. Evidently heat destruction during the condensing and evaporating processes was responsible for the losses. Both heat destruction during concentration and metabolic destruction by starter organisms could have been responsible for the low vitamin B_{12} potency of yoghurt.

When the foods assayed were classified into a few categories on the basis of their vitamin B_{12} potencies, among those having less than 1 microgram per 100 grams, moist-basis, were the fluid-milk products—buttermilk, yoghurt, and condensed, evaporated, and pasteurized whole milk;

certain cheeses—cream, Cheddar, cottage, and Roquefort-blue; and chicken and pork muscle cuts. In the group with 1 to 3 micrograms per 100 grams were Camembert and Limburger cheeses; various kinds of frankfurters; beef, veal, and lamb muscle cuts; and certain seafoods—lobster, scallops, flounder, haddock, swordfish, and tuna. In the group with 3 to 10 micrograms per 100 grams were nonfat dry milk; the seafoods—crabs, rockfish, salmon, and sardines; and egg yolk and dried whole egg. In the group with more than 10 micrograms per 100 grams were clams, oysters, liverwurst, and a variety of organ meats.

Mean vitamin B_{12} values calculated on a moist basis are reported in table 1, together with their standard errors. Although the corresponding standard errors for the dry-basis means are omitted, the ratio of standard error of mean to mean is the same for both moist- and dry-basis values; consequently, those for the latter can be computed without difficulty.

Numbers of brands or sources listed in column 2, table 1, refer in each instance to a single lot of each brand or from each source. For example, 4 sources means a composite of 4 lots each purchased from a different supplier.

Column 13, table 1, which lists vitamin B_{12} values reported by other laboratories, includes those obtained by various microbiological as well as biological methods. This tabulation was not intended to be complete.

In view of the reported role of vitamin B_{12} in the incorporation of amino acids into protein (*28*), investigation was made of the relationship between concentrations of vitamin B_{12} and protein in the foods analyzed. The correlation coefficient computed from the values in columns 5 and 7, table 1, was a negligibly low value, −0.02.

Since the seven lamb legs tested were from animals of known and varying ages, it was considered worthwhile to test the correlation between age and vitamin B_{12} potency from the data of columns 2 and 7, table 1. A significant and high positive correlation coefficient, 0.89, was found. This finding can only be considered suggestive in view of the small number of samples upon which it was based.

SUMMARY

The edible portions of 94 food samples were analyzed by standardized methods for proximate composition and vitamin B_{12} potency. All of them were assayed for vitamin B_{12} by the *Lactobacillus leichmannii* method, and 27 of them by the *Ochromonas malhamensis* method. Both methods are described in detail. The results of the determinations are presented in tabular form.

The precision of each of the two vitamin B_{12} methods was assessed in terms of the mean percentage deviation of the 95-percent confidence limits from each sample mean. In parallel assays, higher vitamin B_{12} values were obtained by the *O. malhamensis* method for 15 of 27 foods tested and by the *L. leichmannii* method for 1 of the 27. Although there was no evidence by which either method could be adjudged as yielding more correct vitamin B_{12} values, the *L. leichmannii* method, on the basis of greater precision and ease of execution, was considered preferable for the assay of foods of animal origin.

A negligibly low negative correlation was found between the vitamin B_{12} and nitrogen contents of the samples.

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Table 1.—*Distribution of vitamin B_{12} in and*

Food	Sample composition	Proximate composition			
		Moisture	Fat	Protein [1]	Ash
		Mean	Mean	Mean	Mean
(1)	(2)	(3)	(4)	(5)	(6)
Dairy Products		*Percent*	*Percent*	*Percent*	*Percent*
Cheese:					
Camembert	2 brands	51.4	22.9	18.8	3.8
Cheddar	3 brands	33.8	30.1	27.9	3.8
Cottage	4 brands	79.0	3.9	12.5	1.2
Cream	3 brands	52.2	35.0	7.6	1.4
Limburger	2 brands	49.1	26.9	19.4	3.9
Roquefort-blue	3 brands	39.1	29.2	22.1	6.6
Swiss:					
Domestic	3 brands	36.5	27.9	28.9	3.3
Imported	1 brand	32.8	32.6	29.8	3.5
Do	1 brand	33.9	30.5	29.1	3.5
Do	1 brand	32.6	30.9	29.4	3.8
Milk:					
Buttermilk, cultured	3 brands	89.4	1.3	3.3	.8
Condensed	1 brand	26.8	8.5	8.1	1.8
Evaporated	4 brands	73.6	7.4	7.2	1.6
Nonfat, dry	1 brand	1.2	.7	36.4	8.1
Do	1 brand	2.4	.4	33.2	8.3
Do	1 brand	2.3	.4	33.4	7.8
Do	1 brand	3.2	.4	33.4	8.1
Do	1 brand	2.5	.4	35.4	8.2
Whole pasteurized	4 brands	87.8	3.4	3.1	.7
Yoghurt	1 brand	86.2	1.6	4.5	1.0
Meats [3]					
Beef:					
Brain	3 sources	78.8	8.9	10.7	1.5
Chuck	4 sources	72.0	5.7	20.6	1.0
Corned	1 sample	64.2	9.4	22.5	3.4
Corned, canned	4 brands	53.7	15.5	27.5	3.1
Dried	4 brands	54.4	5.1	30.2	9.0
Frankfurters	1 brand	54.1	29.4	13.0	2.8
Do	1 brand	60.2	21.7	14.0	2.7
Do	1 brand	59.6	19.9	15.3	3.0
Hamburger	4 sources	63.7	15.0	19.8	1.0
Heart	3 sources	76.6	2.2	18.2	1.2
Kidney	4 sources	77.4	1.2	17.7	1.2
Liver	4 sources	68.1	2.5	20.4	1.5
Rib roast	4 sources	68.0	9.1	20.9	1.0
Roast, canned	4 brands	63.9	6.8	26.7	2.3
Round steak	4 sources	72.6	3.9	21.4	1.0
Stew, canned	4 brands	81.2	3.0	6.4	1.6
Strained, canned	4 brands	78.1	4.7	15.6	1.3
Thymus	3 sources	80.5	1.4	16.1	1.7

See footnotes at end of table.

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proximate composition of foods of animal origin

Vitamin B_{12} potency							
L. leichmannii method			*O. malhamensis* method			Values from other laboratories	
Moist basis		Dry basis	Moist basis		Dry basis	Moist basis	Reference number
Mean	Standard error of mean	Mean	Mean	Standard error of mean	Mean	Range	
(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	
1. 21	0	2. 49	----------	----------	----------	----------	
. 990	. 0060	1. 50	1. 17	0. 0245	1. 77	0. 9 – 2. 23	*20, 33*
. 586	. 0075	2. 79	----------	----------	----------	. 73 – . 95	*20*
. 218	. 0005	. 456	----------	----------	----------	. 2	*24*
1. 04	. 0245	2. 04	1. 01	. 0640	1. 98	----------	
. 587	. 0285	. 964	. 664	. 0075	1. 09	----------	
1. 65	. 0300	2. 60	1. 71	. 0548	2. 69	. 9 – 3. 62	*20, 24,*
. 850	. 0045	1. 26	----------	----------	----------	2. 5 – 3. 2	*22*
1. 10	. 0450	1. 66	----------	----------	----------	----------	
1. 11	. 0250	1. 65	----------	----------	----------	----------	
. 198	. 0046	1. 87	. 240	. 0135	2. 26	----------	
. 308	. 0035	. 421	----------	----------	----------	. 30 – . 63	*10*
. 132	. 0060	. 500	----------	----------	----------	. 10 – . 3	*10, 24*
3. 76	. 0245	3. 81	4. 92	. 135	4. 98	2. 5 – 4. 2	*21, 24*
3. 11	. 0250	3. 19	----------	----------	----------	----------	
3. 59	. 100	3. 67	----------	----------	----------	----------	
3. 63	. 0900	3. 75	----------	----------	----------	----------	
4. 01	. 205	4. 11	----------	----------	----------	----------	
. 362	. 0235	2. 97	----------	----------	----------	. 16 – . 95	*11, 12, 21, 24*
. 0809	. 0060	. 586	. 0601	. 0006	. 436	[2] . 0174– . 0825	*14*
7. 83	. 340	36. 9	----------	----------	----------	4. 5 – 4. 9	*44*
2. 17	. 0250	7. 75	----------	----------	----------	----------	
. 846	. 0470	2. 36	----------	----------	----------	----------	
1. 84	. 0550	3. 97	----------	----------	----------	----------	
1. 84	. 0350	4. 04	----------	----------	----------	----------	
1. 36	. 0050	2. 96	----------	----------	----------	----------	
1. 36	. 0350	3. 42	----------	----------	----------	----------	
1. 47	. 0200	3. 64	----------	----------	----------	----------	
1. 91	. 0050	5. 26	----------	----------	----------	----------	
11. 3	. 361	48. 3	14. 2	. 600	60. 7	8. 8 – 10. 6	*44*
38. 3	. 900	169.	----------	----------	----------	14. 6 – 55	*33, 42, 43, 44*
116.	3. 85	364.	122.	5. 00	382.	15 –130	*17, 18, 24, 33, 42, 43, 44, 45*
1. 24	. 0300	3. 88	----------	----------	----------	2. 5 – 2. 9	*42*
2. 25	. 0050	6. 23	----------	----------	----------	1. 0 – 2. 5	*24*
1. 93	. 0447	7. 04	2. 06	. 0321	7. 52	1. 3 – 7. 5	*33, 40, 42, 43*
. 647	. 0100	3. 44	----------	----------	----------	----------	
1. 50	. 0500	6. 85	----------	----------	----------	----------	
2. 13	. 0346	10. 9	2. 30	. 115	11. 8	----------	

TABLE 1.—*Distribution of vitamin B_{12} in and proximate*

Food	Sample composition	Proximate composition			
		Moisture	Fat	Protein [1]	Ash
		Mean	Mean	Mean	Mean
(1)	(2)	(3)	(4)	(5)	(6)
MEATS [3]—Continued		*Percent*	*Percent*	*Percent*	*Percent*
Lamb:					
Kidney	4 sources	78. 8	2. 2	16. 0	1. 2
Leg	1 sample; age of animal, 3 to 4 months.	72. 9	5. 4	19. 8	1. 1
Do	1 sample; age of animal, 7 months.	75. 5	3. 1	19. 5	1. 1
Do	1 sample; age of animal, 8 months.	72. 3	5. 3	20. 7	1. 0
Do	1 sample; age of animal, 19 months.	70. 4	8. 4	19. 7	1. 0
Do	1 sample; age of animal, 20 months.	71. 8	6. 5	20. 2	1. 0
Do	1 sample; age of animal, 80 months.	71. 6	5. 7	21. 3	1. 0
Do	1 sample; age of animal, 80 months.	71. 9	6. 7	19. 4	1. 0
Liver	4 sources	64. 7	5. 6	20. 9	1. 4
Stew meat	4 sources	69. 3	9. 0	19. 8	1. 0
Mixed:					
Frankfurters:					
All-meat	1 brand	51. 9	30. 5	13. 2	3. 0
Do	1 brand	51. 5	31. 8	12. 2	2. 1
Do	1 brand	51. 9	29. 4	12. 8	2. 7
Do	1 brand	58. 2	22. 4	14. 2	3. 0
Meat and milk solids	1 brand	56. 2	22. 8	13. 4	3. 1
Lebanon bologna	4 brands	50. 3	25. 1	17. 2	4. 6
Liverwurst	4 brands	50. 8	27. 4	14. 4	3. 2
Pork:					
Ham, cured	3 sources	63. 1	6. 1	23. 4	6. 6
Loin	1 sample	70. 6	4. 9	22. 9	1. 1
Do	1 sample	69. 5	7. 6	21. 0	1. 1
Do	1 sample	69. 6	7. 8	21. 4	1. 1
Do	1 sample	69. 0	7. 6	22. 3	1. 0
Shoulder	1 sample	72. 4	5. 9	20. 6	1. 0
Sausage	4 brands	39. 1	47. 5	10. 2	2. 3
Veal:					
Chop	4 sources	73. 7	2. 0	22. 0	1. 2
Steak	4 sources	74. 5	1. 1	22. 1	1. 1
Stew meat	4 sources	74. 2	1. 0	22. 8	1. 0
POULTRY PRODUCTS [3]					
Chicken:					
Boned, canned	3 brands	65. 2	10. 2	23. 1	1. 6
Breast	4 sources	74. 3	. 9	22. 4	. 9
Egg:					
White	48 eggs	88. 7	0	9. 7	. 5
Yolk	48 eggs	48. 7	31. 3	16. 4	1. 7
Whole	Calculated from white and yolk.	75. 4	10. 4	11. 9	. 9
Do	24 eggs	74. 3	11. 6	12. 6	. 9
Whole dried	1 sample	1. 8	39. 4	45. 5	3. 4
Gizzard	15 pieces	78. 6	1. 3	18. 6	1. 0
Heart	4 sources	77. 3	5. 0	15. 4	. 8
Liver	25 pieces	71. 3	2. 4	18. 9	1. 3
Thigh	4 sources	74. 6	4. 6	19. 0	. 9

See footnotes at end of table.

Vitamin B_{12} potency							
L. leichmannii method			*O. malhamensis* method			Values from other laboratories	
Moist basis		Dry basis	Moist basis		Dry basis	Moist basis	Reference number
Mean	Standard error of mean	Mean	Mean	Standard error of mean	Mean	Range	
(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	μg./100 g.	
63. 0	0. 650	297.	----------	----------	----------	----------	
1. 29	. 0500	4. 76	----------	----------	----------	1. 7 – 7. 4	*42, 43*
1. 93	. 0450	7. 88	----------	----------	----------	----------	
1. 40	. 0245	5. 05	1. 63	0. 000	5. 88	----------	
1. 78	. 0900	6. 01	2. 04	. 165	6. 89	----------	
1. 78	. 0300	6. 31	----------	----------	----------	----------	
2. 34	. 0300	8. 24	3. 10	. 0949	10. 9	----------	
2. 49	. 0300	8. 86	----------	----------	----------	----------	
104.	. 500	295.	----------	----------	----------	39 –110	*2*
2. 43	. 0100	7. 92	----------	----------	----------	----------	
1. 12	. 0100	2. 33	----------	----------	----------	----------	
1. 19	. 0250	2. 45	----------	----------	----------	----------	
1. 29	. 0150	2. 68	----------	----------	----------	----------	
1. 54	. 105	3. 68	----------	----------	----------	----------	
1. 68	. 105	3. 84	----------	----------	----------	----------	
1. 97	. 120	3. 96	1. 90	. 185	3. 82	----------	
13. 9	. 550	28. 3	----------	----------	----------	----------	
. 586	. 0195	1. 59	. 797	. 0055	2. 16	. 9 – 2. 9	*33, 42, 43, 49*
. 555	. 0160	1. 89	. 699	. 0165	2. 38	. 5 – . 8	*30*
. 374	. 0055	1. 23	----------	----------	----------	----------	
. 416	. 0020	1. 37	----------	----------	----------	----------	
. 465	. 0155	1. 50	----------	----------	----------	----------	
. 562	. 0115	2. 04	----------	----------	----------	[2] 1. 0	*40*
. 536	. 0015	. 880	----------	----------	----------	1. 5	*24*
1. 58	. 0850	6. 01	----------	----------	----------	----------	
1. 70	. 0350	6. 67	----------	----------	----------	----------	
2. 01	. 0100	7. 79	----------	----------	----------	----------	
. 794	. 0165	2. 28	----------	----------	----------	----------	
. 415	. 0030	1. 61	. 498	. 0415	1. 94	----------	
. 0909	. 0006	. 804	----------	----------	----------	. 009 – . 063	*16, 36*
9. 26	. 0500	18. 1	----------	----------	----------	. 28 – 3	*16, 33, 36*
3. 13	----------	6. 54	----------	----------	----------	. 607 – 10. 4	*16, 46*
. 837	. 0315	3. 26	----------	----------	----------	----------	
10. 0	. 300	10. 2	12. 1	. 346	12. 3	----------	
2. 31	. 0141	10. 8	3. 19	. 0493	14. 9	----------	
3. 75	. 125	16. 5	----------	----------	----------	----------	
22. 3	1. 20	77. 7	24. 1	. 245	84. 0	11 – 27	*33, 45*
. 359	. 0240	1. 41	. 473	. 0050	1. 86	----------	

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